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Formation of hematopoietic territories and bone by transplanted human bone marrow stromal cells requires a critical cell density

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Objective. **Bone marrow stromal cells (BMSCs) include multipotent cells with the ability to form mature bone organs upon in vivo transplantation. Hematopoiesis in these bone organs has been ascribed to the action of skeletal stem cells, which are capable of differentiating towards bone and hematopoiesis-supporting stroma. Yet, the creation of hematopoietic territories may be in part a natural consequence of the formation of a sufficiently mature and large bone microenvironment. Here, we describe, for the first time, a relationship between BMSC numbers and the extent of bone/hematopoiesis formation in heterotopic transplants.**

Methods. **Human BMSCs were transplanted along with hydroxyapatite/tricalcium phosphate, utilizing a spectrum of dosages, into immunotolerant mice; the transplants were followed for up to 29 months.**

Results. **The extent of bone and hematopoiesis formation increased with increasing BMSC numbers; however, the relationship was sigmoid in character, and a threshold number of BMSCs was necessary for extensive bone formation or any hematopoiesis. Hematopoiesis only occurred in conjunction with extensive bone formation, and no hematopoiesis occurred where bone formation was poor. Consistent with our earlier studies of long-term BMSC transplantation, the transplants underwent a change in bone morphology but not bone content after 8 weeks.**

Conclusion. **Our results have provided evidence that the formation of both hematopoiesis and a mature bone organ is as much a consequence of a sufficiently high local density of bone marrow stromal cells as it is the product of skeletal stem cell action.** © **2007 International Society for Experimental Hematology. Published by Elsevier Inc.**

The creation of mature bone organs remains an active challenge for bone scientists and reconstructive surgeons. The components of a mature bone include cortical and cancellous structures, vasculature, a regenerative lining, and a marrow space comprised of hematopoietic elements and stroma. Mature bone or a bone and marrow organ have been successfully recapitulated in animal models by the transplantation of populations of culture-expanded osteoprogenitor cells, such as bone marrow stromal cells (BMSCs) [1–4].

BMSCs contain a subset of the multipotent nonhematopoietic cells resident in the bone marrow. These cells, first described by Friedenstein and Owen in the early 1970s, were isolated by virtue of their high adherence to tissue culture plastic and glassware, and proliferated extensively in vitro [1,4]. BMSCs are capable of differentiating into mesenchymal tissue elements, including bone, cartilage, hematopoiesis-supporting stroma, and adipose [1–4]. Additional studies have suggested the possibility that BMSCs may differentiate into striated muscle, smooth muscle, hepatic, and even pancreatic elements [5,6]. In our hands, stromal cells refer to adherent colony-forming units (fibroblast) (CFU-f) recovered from the bone marrow. These cells have been characterized as expressing markers for collagen I and III, CD44, osteonectin, and CD106, and for not expressing markers to CD34 or Factor VIII [7].

Adherent BMSCs include a heterogenous population of clonogenic cells. When primary BMSC clones are expanded in tissue culture and then individually transplanted

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into syngeneic or immunocompromised recipients, they form three types of transplants: non–bone-forming, bone-forming without hematopoiesis, and bone-forming with hematopoiesis, in which hematopoietic cells of recipient origin populate hematopoiesis-supporting stroma formed by donor-derived BMSCs [8]. In such transplants, hematopoiesis without bone formation has never been observed [8,9]. Among bone-forming transplants, it is presently unclear why some BMSC clones form a mature hematopoietic organ while others do not. Two mutually compatible theories may explain this phenomenon.

First, it has been hypothesized that the presence of hematopoiesis-supportive stroma within the transplants reflects the activity of BMSCs with a self-regenerative and multipotential capacity; these cells have been termed skeletal stem cells (SSCs) [10]. Here, SSCs refer to a population of cells derived from the bone marrow with the potential to form both bone and hematopoietic territories in vivo, a characteristic not shared by all marrow stromal cells or by all mesenchymal stem cells. In contrast, the absence of hematopoiesis in the presence of new bone is considered to be the product of BMSCs that are already committed to a bone-forming lineage; these cells have been called committed osteoprogenitors (OPs). While no phenotypic marker is available to distinguish SSCs from OPs, individual clones of these cell types form hematopoietic and nonhematopoietic bone, respectively, when transplanted in vivo.

A second possible explanation for the presence of hematopoiesis in only some BMSC transplants is that hematopoiesis only occurs when a significant amount of bone has formed, and only when this bone is sizable enough to encapsulate a hematopoietic space. The basis for this hypothesis lies in our observation, following analyses of over 2300 human BMSC transplants placed into mice, that hematopoiesis, while always accompanying bone, has rarely occurred in human BMSC transplants where only modest bone has formed, and likewise only rarely fails to form where bone formation is significant (Mankani, unpublished data).

The primary purpose of this study was to test the hypothesis that hematopoiesis in BMSC transplants is the product of extensive bone formation in addition to the action of SSCs. Towards this end, human BMSCs were co-transplanted with hydroxyapatite/tricalcium phosphate (HA/TCP) particles at various dosages into mouse recipients, and were harvested at time points ranging from 7 to 123 weeks. The quantity of bone and hematopoiesis as a function of transplant interval and cell dosage were determined.

This study also had a secondary purpose, in that it offered technical information for optimizing BMSC transplantation. When human BMSCs are transplanted for the purpose of engineering new bone, best results can be achieved if the cells are combined with HA/TCP particles [11]. Many parameters of BMSC cultivation and transplantation have been optimized by our group and others, including media and serum requirements, optimal HA/TCP particle size, methods to deliver cell/particle combination into the area needing new bone, and ways to noninvasively estimate the extent of new bone formation [8,12–16]. Up to now, however, no study has determined the optimum density of BMSCs relative to HA/TCP matrix needed to achieve adequate bone formation. For therapeutic transplantation, billions of autologous BMSCs have to be generated in vitro when starting with a bone marrow aspirate. If BMSCs are then transplanted at a lower than optimum density, bone formation will be poor; if BMSC density is greater than optimum, we would anticipate cell wastage and prolonged cell expansion times. Thus, information on the optimum BMSC density is absolutely necessary if human autologous BMSCs are to be used for large-scale therapeutic transplantation.

Methods

Transplant preparation, placement, and recovery

Surgical specimens were obtained as fragments of normal unaffected bone with bone marrow from patients undergoing reconstructive surgery. The patients were a girl aged 14 years and a boy aged 13 years undergoing iliac crest bone harvest for correction of scoliosis. One of these patients' cells were also used in a separate study [17], but none of the transplants or animals were shared between the two studies. Tissue procurement proceeded in accordance with institutional regulations governing the use of human subjects, including the use of informed consent. We generated multicolony-derived strains of BMSCs from the bone marrow in a manner previously described [8]. In brief, a single cell suspension of bone marrow cells was cultured in growth medium consisting of α-MEM (Invitrogen, Grand Island, NY, USA), 2 mM L-glutamine, 100 U/mL penicillin, 100 ug/mL streptomycin sulfate (Biofluids, Rockville, MD, USA), 10^{-8} M dexamethasone (Sigma, St. Louis, MO, USA), 10^{-4} M L-ascorbic acid phosphate magnesium salt n-hydrate (Wako, Osaka, Japan), and 20% fetal bovine serum of a preselected lot (Equitech-Bio, Kerrville, TX, USA). In our hands, the use of dexamethasone and ascorbic acid phosphate stimulates cell proliferation but has a negligible effect on the differentiation of transplanted BMSCs. The cells were incubated at 37°C in an atmosphere of 100% humidity and 5% CO_2. Cells were passaged at near confluence with Trypsin-EDTA (Invitrogen, Carlsbad, CA, USA).

Upon reaching confluence at the final passage (passages 2 through 5), cells were trypsin-released and pipetted into 1.8-mL polypropylene cryotubes (Nunc, Roskilde, Denmark), each previously loaded with a 40-mg aliquot of HA/TCP particles (Zimmer, Warsaw, IN, USA). Consistent with our previous determinations of optimum particle size [14], only particles of size range 0.5 to 1.0 mm were isolated with a sieve shaker (CSC Scientific, Fairfax, VA, USA) and used in these experiments. Each tube received one of the following doses of cells: 3.0 million, 1.0 million, 0.3 million, 0.1 million, 0.03 million, or no cells, in 1 mL of growth medium. The mixtures were incubated for 90 minutes at 37°C on a slowly rotating platform. They were then centrifuged at 200*g* for 60 seconds, and the supernatant was discarded. An

approximately equal number of transplants were generated from cells from each of the 2 donors.

Three-month-old immunocompromised Bg-Nu/Nu-Xid female mice (Harlan-Sprague Dawley, Indianapolis, IN, USA) served as transplant recipients. All animals were cared for according to the policies and principles established by the Animal Welfare Act and the NIH Guide for the Care and Use of Laboratory Animals. Operations were performed in accordance to specifications of an approved institutional small-animal protocol. Mice were anesthetized with a combination of intraperitoneal (IP) ketamine (140 mg/kg body weight) and IP Xylazine (7 mg/kg body weight). Transplants were placed in the subcutaneous tissues beneath the dorsal skin through a midline longitudinal skin incision. Incisions were closed with stainless steel surgical staples (Roboz Surgical Instrument Co., Inc., Rockville, MD, USA). Each mouse received either 5 or 6 transplants; all mice received at least 5 transplants, each transplant reflecting one of the 5 doses which included BMSCs, and some mice also received a sixth transplant containing no BMSCs. Each transplant was placed sufficiently far from its neighbors to not have a direct or identifiable effect. This has been confirmed in our previous studies, where transplants with cells derived from normal and diseased donors were always placed in the same mouse (in order to avoid recipient variability), and yet with each transplant developing bone specific to its donor, without apparent interference or contamination by cells from the other transplants [18]. Twenty-nine mice were transplanted with 151 transplants. Of these, 20 mice survived the experiments and 101 transplants were recovered; these were harvested at time points ranging from 7 to 123 weeks (29 months) postoperatively.

The transplants were fixed in 4% phosphate-buffered formalin freshly prepared from paraformaldehyde (PBF) (Sigma Chemical Co, St. Louis, MO, USA). Following an overnight fixation at 4°C, the transplants were suspended in phosphate-buffered saline (PBS) (Invitrogen). The transplants were completely demineralized prior to embedding in buffered 10% EDTA (Quality Biological, Inc., Gaithersburg, MD, USA). Each disc-shaped transplant, measuring approximately 8 mm in diameter, was divided into four pieces using 3 parallel cuts. These were embedded in paraffin so that their largest cut surfaces were sectioned. A set of at least 8 such 7-micron sections was obtained from each transplant. Sections were deparaffinized, hydrated, and stained with hematoxylin and eosin (H&E). Separate unstained slides, incorporating 5-micron-thick sections, were prepared for in situ hybridization, described below.

Estimation of bone formation and hematopoiesis

The H&E-stained sections were examined histologically, and the extent of bone within each transplant was scored on a semiquantitative scale by 3 independent, blinded observers in a manner similar to that described previously [8,14,15]. Each observer was an investigator in our laboratory who had been trained to evaluate the histologic characteristics of the transplants. Transplants were scored on a scale of 0 to 4; a score of 0 corresponded to no bone formation, while a score of 4 was given to transplants with abundant bone formation occupying greater than one half of the section (Table 1 and Fig. 1). The data obtained were analyzed statistically using JMPIN version 4.0.4 (SAS Institute, Cary, NC, USA). Additionally, each transplant was assessed histologically for either the presence or absence of hematopoiesis. Hematopoiesis was confirmed by the presence of megakaryocytes, which are resident within bone marrow but are not found in either fibrovascular tissue or the peripheral circulation (Fig. 1F).

Table 1. Semi-quantitative scale for the estimation of bone formation

Score	Extent of bone present within the transplant
0	No bone evident
1	Minimal bone evident (1 trabecula)
2	Weak bone formation, occupying only a small portion of the section
3	Moderate bone formation, occupying a significant portion but less than one half of the section
4	Abundant bone formation, occupying greater than one half of the section

Quantification of cells per particle

We sought to confirm that BMSCs attached to the HA/TCP particles in numbers proportional to their loading doses. Random aliquots of particles with attached BMSCs were collected from cryotubes in which BMSCs had been incubated with HA/TCP particles for 90 minutes. Prior to collection, the media was discarded and the particles were rinsed twice with α-MEM, to remove all cells which had not already bound to particles. Single particles were then removed using a sterile jeweler's forceps and each particle plated into its own well of a 96-well plate (Becton-Dickinson Labware, Franklin Lakes, NJ, USA) along with 100 mL of growth medium.

The resulting cell cultures were fixed on day 7 or 8 with absolute methanol and stained with an aqueous solution of saturated methyl violet (Sigma). Clusters of BMSCs containing 5 or more cells were counted using a dissecting microscope, and the number of clusters per particle was calculated. The percentages of cells adhering to HA/TCP particles were analyzed using a one-way analysis of variance model. All statistical analyses were performed with the SAS software (JMPIN version 4.0.4; SAS Institute, Cary, NC, USA).

Quantification of donor cells

We then sought to describe the number of human cells within a representative sample of transplants, to determine whether the numbers of human BMSCs and their descendants in the mature transplants were equivalent to the original dosage. The origin of bone-forming cells within sets of transplants from 2 of the mice harvested at 9 weeks was confirmed through in situ hybridization. The results were quantified in order to determine whether the numbers of human cells in the transplants with a low BMSC dosage were proportionally diminished in comparison to those transplants with a high BMSC dosage. Unstained sections of slides from all 6 transplants of the mice were obtained as described above. Control slides consisting of human and mouse tissue underwent in situ hybridization with the probe during each hybridization run, serving as positive and negative controls, respectively.

The human-specific repetitive *alu* sequence, which comprises about 5% of the total human genome, was applied for identification of human cells [19]. We used in situ hybridization for the *alu* sequence to study the origin of tissues formed in the transplants. The digoxigenin-labeled probe specific for the *alu* sequence had been prepared by PCR, including 1× PCR buffer

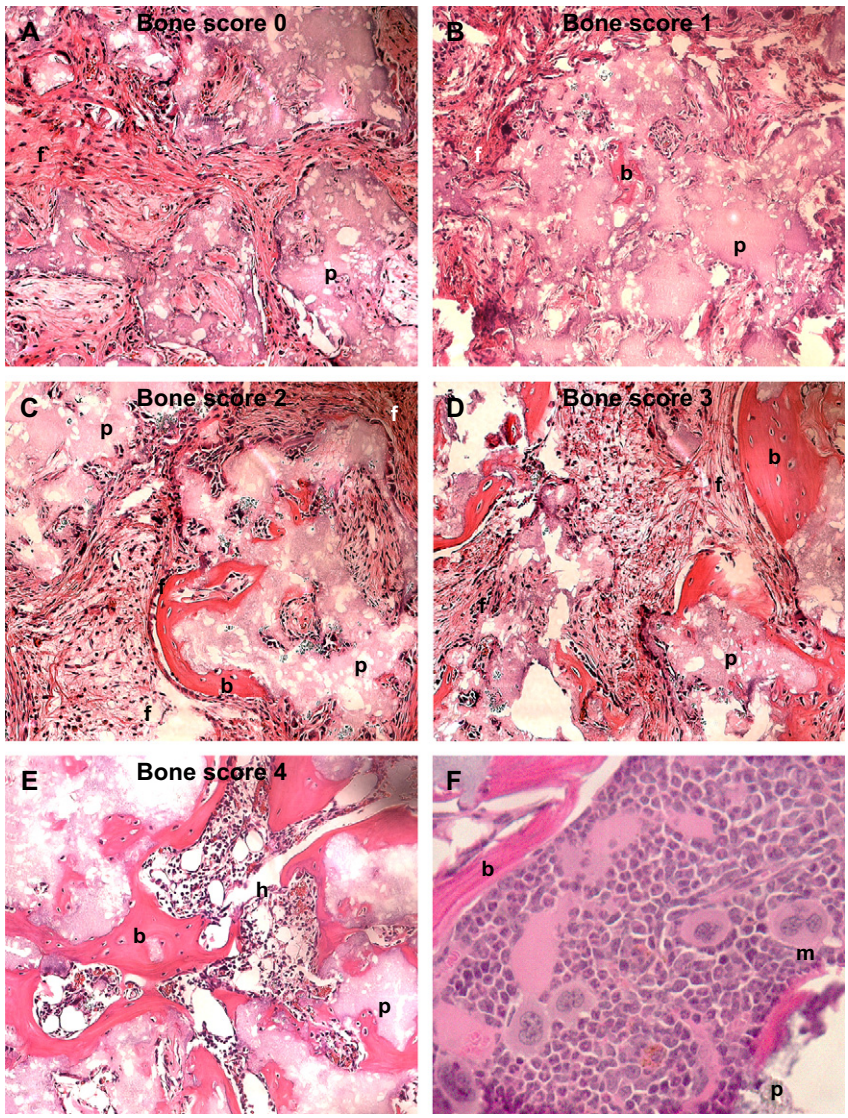

Figure 1. HA/TCP and BMSC transplants that have formed varying amounts of bone. **(A)** Transplant exemplifying a bone score of 0. No bone formation present. Rather, particles are widely separated by connective tissue. **(B)** Transplant exemplifying a bone score of 1. Only 1 bone trabecula present. **(C)** Transplant exemplifying a bone score of 2. Weak bone formation, with only a few trabeculae present. New bone does not bridge adjacent particles. **(D)** Transplant exemplifying a bone score of 3. Bone formation is appreciable but less than one half of the transplant. **(E)** Transplant exemplifying a bone score of 4. Abundant bone formation. Bone bridges adjacent particles. **(F)** Megakaryocyte within a hematopoietic pocket in a transplant. b, bone; f, fibrous connective tissue; p, particle; h, hematopoietic tissue; m, megakaryocyte. Magnification: 10× (A–E), 40× (F). Stain: Hematoxylin and eosin; paraffin embedding following demineralization.

(Perkin Elmer, Foster City, CA, USA), 0.1 mM dATP, 0.1 mM dCTP, 0.1 mM dGTP, 0.065 mM dTTP, 0.035 mM digoxigenin-11-dUTP (Boehringer Mannheim Corp., Indianapolis, IN, USA), 10 pmol of specific primers, and 100 ng of human genomic DNA. The following primers were used on the basis of previously reported sequences [20]: sense, 5′-GTGGCTCACGCCTGTAATCC-3′, and antisense, 5′-TTTTTTGAGACGGAGTCTCGC-3′. The method for in situ hybridization of HA/TCP containing transplants has been previously described [8]. Sections deparaffinized with xylene and ethanol were immersed in 0.2 N HCl at room temperature for 7 minutes and then incubated in 1 mg/mI pepsin in 0.01 N HCl at 37°C for 10 minutes. After washing in PBS, the sections were treated with 0.25% acetic acid containing 0.1 M triethanolamine (pH 8.0) for 10 minutes and prehybridized with 50% deionized formamide containing 4× SSC at 37°C for 15 minutes. The sections were then hybridized with 1 ng/uL digoxigenin-labeled probe in hybridization buffer (1× Denhardt's solution, 5% dextran sulfate, 0.2 mg/mI salmon sperm DNA, 4× SSC, 50% deionized formamide) at 42°C for 3 hours following a denaturation step at 95°C for 3 minutes. After washing with 2× SSC and 0.1× SSC, digoxigenin-labeled DNA was detected by immunohistochemistry using antidigoxigenin alkaline phosphatase–conjugated Fab fragments (Boehringer Mannheim Corp., Mannheim, Germany).

Following in situ hybridization, we counted the total number of *alu*-positive cells in each tissue section. The total area of each

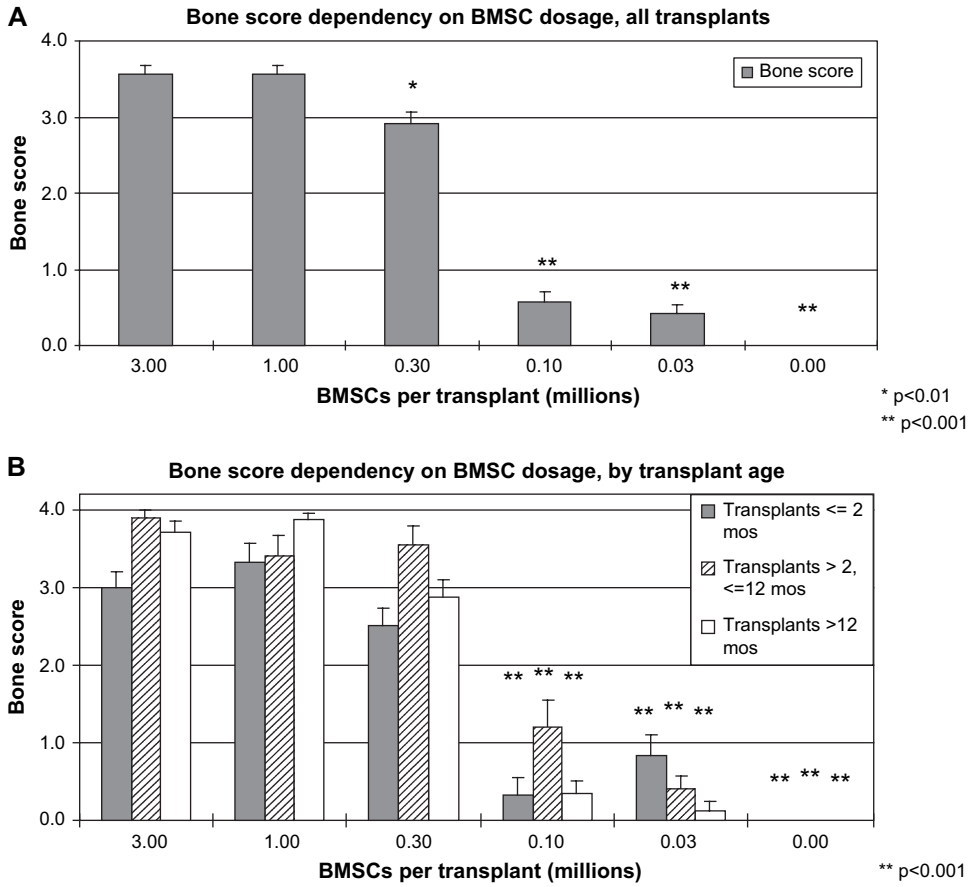

Figure 2. **(A)** For all transplants, regardless of time of harvest, bone score increased significantly with increasing BMSC dose, up to 1.0 million cells per transplant. Bone scores for 1.0 and 3.0 million cell transplants were equivalent. *p* values are relative to the group of 3.0 million cells per transplant. **(B)** Bone score increased only incrementally among BMSC transplants as they were harvested at later time points.

section was determined using digitized microscopy and imaging software (Adobe Photoshop version 5.0; Adobe Systems Inc., San Jose, CA, USA), to arrive at a ratio of *alu*-positive cells per square millimeter of transplant section.

Results

Twenty mice with 101 transplants were euthanized starting at 4 weeks posttransplantation. Only mice 4 or more weeks posttransplantation were analyzed, because human BMSCs transplants produce nearly no bone prior to that time point (unpublished data). Transplants were harvested as late as 29 months following placement.

Timing and extent of bone formation

Bone formation increased with increasing cell dose (Fig. 2A and B). A sharp increase in bone formation was associated with an increase in dose from 0.1 million to 0.3 million cells. Transplants with no BMSCs produced no bone, while transplants with 0.03 million and 0.1 million cells produced minimal bone. As the dose increased to 0.3 million cells, transplants produced weak to moderate amounts of bone; above this dose, moderate to significant amounts of bone were seen.

As transplants matured, the amount of bone did not increase significantly with increasing transplant age among individual dosages. As transplant age increased from less than 2 months to the interval 2 to 12 months, bone score increased slightly among all groups except 0.03 million BMSCs per transplant. However, transplants older than 12 months demonstrated slight reductions in bone score for all groups except 1.0 million BMSCs per transplant.

Transplant morphology

Bone morphology and the spatial arrangement of the bone relative to the HA/TCP particles varied with BMSC dose. Transplants employing the largest BMSC doses (3.0 million and 1.0 million cells) were characterized by extensive bone trabeculae closely associated with individual HA/TCP particles, abundant associated hematopoietic tissue, and occasional adipocytes (Fig. 3). In some areas, bone associated with individual particles appeared to coalesce, forming a rim of bone along the exterior surface of the transplant and a latticework of bone within the transplant. Transplants

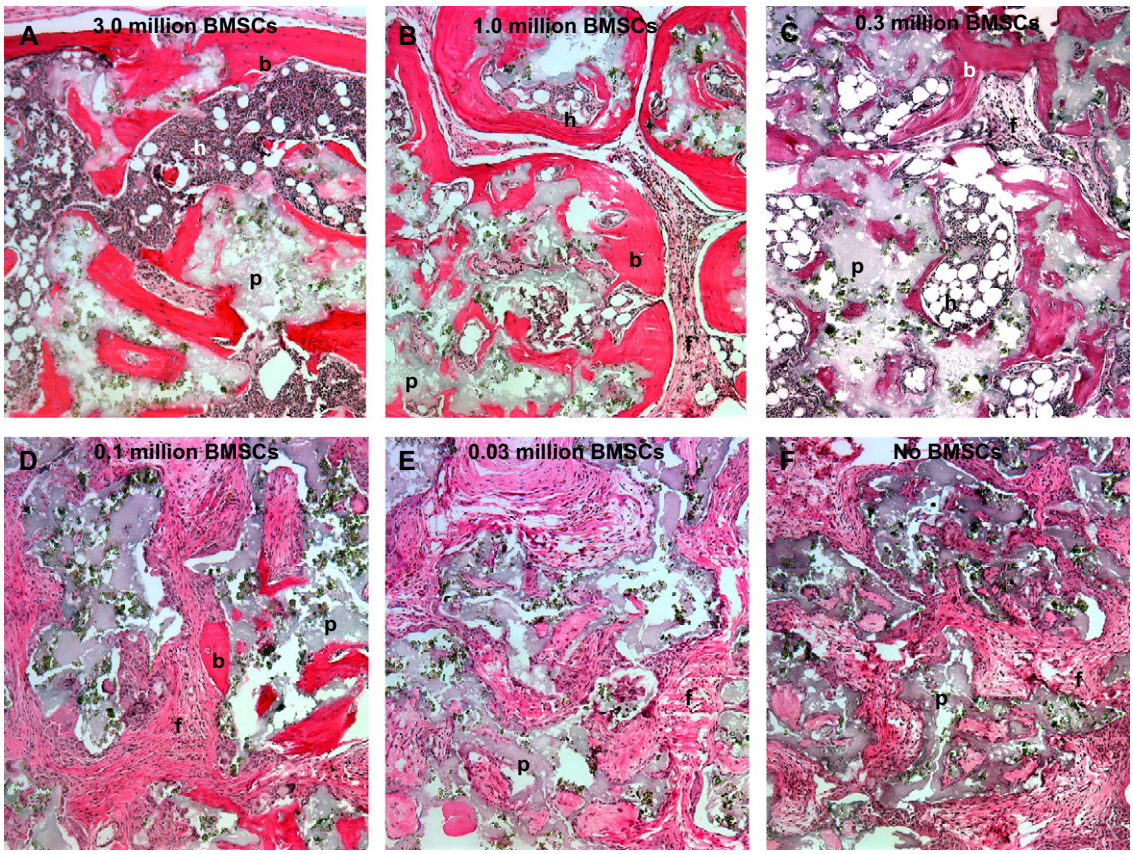

Figure 3. **(A)** 10-week-old, 3-million-BMSC transplant. Note mature cortico-cancellous bone interspersed among HA/TCP particles, and minimal fibrovascular tissue. **(B)** 10-week-old, 1-million-BMSC transplant. Note slightly less bone and greater fibrovascular tissue relative to the transplant in A. **(C)** 10-week-old, 0.3-million-BMSC transplant. Significant bone within the section. **(D)** 10-week-old, 0.1-million-BMSC transplant. **(E)** 10-week-old, 0.03-million-cell transplant. No bone is evident in this section. Fibrovascular tissue widely separates the particles. **(F)** 10-week-old transplant devoid of BMSCs. b, bone; f, fibrous connective tissue; p, particle; h, hematopoietic tissue. Magnification: 10×. Stain: Hematoxylin and eosin; paraffin embedding following demineralization.

with 0.3 million cells had abundant bone and hematopoietic tissue, but bone was interspersed with fibrovascular tissue. Transplants with 0.1 and 0.03 million cells had sporadic bone trabeculae which were uniformly adherent to HA/TCP particles; occasionally, a bone trabecula bridged 2 adjacent particles. No hematopoiesis was evident. Transplants with no BMSCs had no bone formation; fibrovascular tissue filled the spaces between the particles.

Hematopoiesis was present only in transplants with bone score 3 or 4, and no hematopoiesis was present in transplants with bone scores 0, 1, or 2. All hematopoietic spaces were circumscribed by newly formed, BMSC-derived bone in combination with one or more HA/TCP particles (Fig. 4A). Osteoclasts were identified by their size, multinuclear morphology, and a characteristic position within a bony lacunae that they had not created (not shown). Human-derived osteocytes and osteoblasts were identified by their position within and adjacent to the bone, using in situ hybridization with a probe specific for *alu.*

Transplants evolved in appearance the longer they stayed in the mice. Transplants over 104 weeks of age grossly appeared more vascular, likely associated with its more extensive hematopoietic tissue. Histologic examination of these transplants showed that their overall profile was unchanged in comparison with 10-week-old transplants (compare Fig. 4B and C). Among transplants with high BMSC numbers (0.3 to 3.0 BMSCs), a thinner and more extensive latticework of bone was evident in older transplants, with fewer and smaller HA/TCP particles but larger hematopoietic spaces. The bone itself maintained its trabecular, laminar structure, and increasingly appeared to form a cortical shell at the periphery of the transplant. Transplants with few BMSCs did not change appearance significantly between early and late time points (not shown).

Quantification of colony-forming cells per particle

Since bone formation increased nonlinearly with increasing BMSC dose, we sought to determine whether this was due to a nonlinear capture of BMSCs by the HA/TCP particles. Twelve transplants were analyzed, each with a BMSC dose of 0.1, 0.3, or 1.0 million cells (Fig. 4D). In transplants with

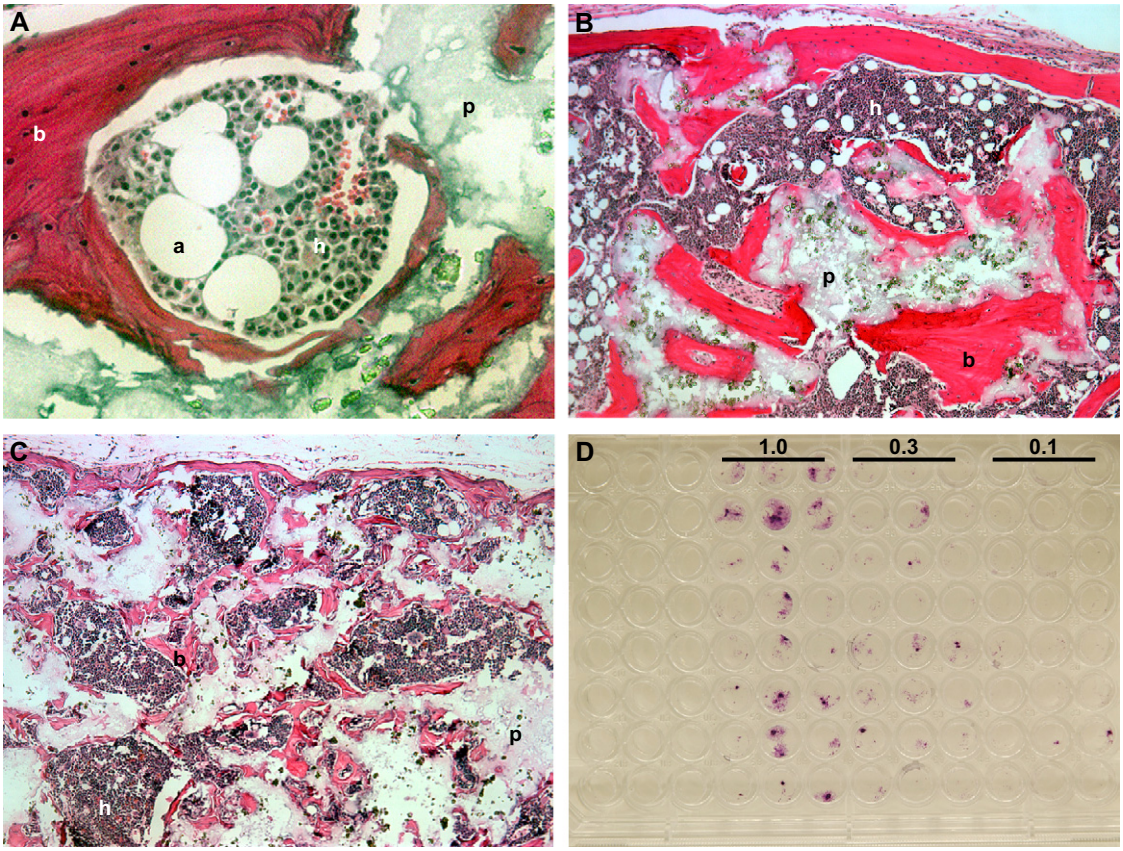

Figure 4. **(A)** 56-week-old, 0.3-million-BMSC transplant. Typical appearance of a hematopoietic space bounded by newly formed, BMSC-derived bone, and an HA/TCP particle. **(B)** 10-week-old, 3-million-BMSC transplant. **(C)** 104-week-old, 3-million-BMSC transplant. In comparison to transplant in (B), the outer rim of cortical bone and the internal trabeculae have thinned. Many of the HA/TCP particles have been partially resorbed, and those spaces have been replaced with hematopoietic elements. **(D)** Photograph of a typical 96-well plate, demonstrating stained BMSC clusters from particles from 3 different transplants, one for each dosage studied (1.0, 0.3, 0.1 million BMSCs each). a, adipocyte; b, bone; f, fibrous connective tissue; p, particle; h, hematopoietic tissue. Magnification: 20× (A), 10× (B,C). Stain: Hematoxylin and eosin; paraffin embedding following demineralization.

0.1 million BMSCs, most particles had no associated cluster of BMSCs in tissue culture. In contrast, particles from transplants with 1.0 million cells were each associated with as many as 4 BMSC clusters. The mean number of clusters per particle for BMSC doses of 0.1, 0.3, and 1.0 million cells per transplant were 0.14, 0.79, and 1.63, respectively ($p < 0.001$ between each pair of groups). Our results suggest that the number of adherent clusters of cells recovered from each particle was roughly proportional to the number of BMSCs placed on the particle initially.

Quantification of donor cells

Since our quantification of adherent clones per particle demonstrated that the particles retained BMSCs in numbers proportional to their original dosage, we then sought to determine whether the numbers of human BMSCs in the mature transplants were also proportional to the original dosages. Eight to 12 sections from each transplant underwent in situ hybridization with an *alu*-specific probe. The location of *alu*-positive cells correlated very well with trabecular bone within the transplants, and no *alu*-positive cells were found independently of the new bone (Fig. 5). As expected, hematopoietic cells were uniformly *alu*-negative, confirming their recipient origin.

The total number of *alu*-positive cells were normalized to the actual area of each transplant (Fig. 6A). Transplants with 3.0 million BMSCs had over 36 *alu*-positive cells per square millimeter of transplant, while transplants with 1.0 and 0.3 million cells had 14.4 and 12.0 probe-positive cells per square millimeter, respectively. Correspondingly, transplants with few BMSCs had few probe-positive cells. When the normalized number of *alu*-positive cells in the transplants were compared to the number of BMSCs in the original transplants (Fig. 6B), transplants with 0.3 million BMSCs retained the highest proportion of donor cells or their progeny, while transplants with higher BMSC dosages showed a decrease of retained BMSCs. Transplants with low BMSC doses retained an intermediate proportion of their original BMSCs.

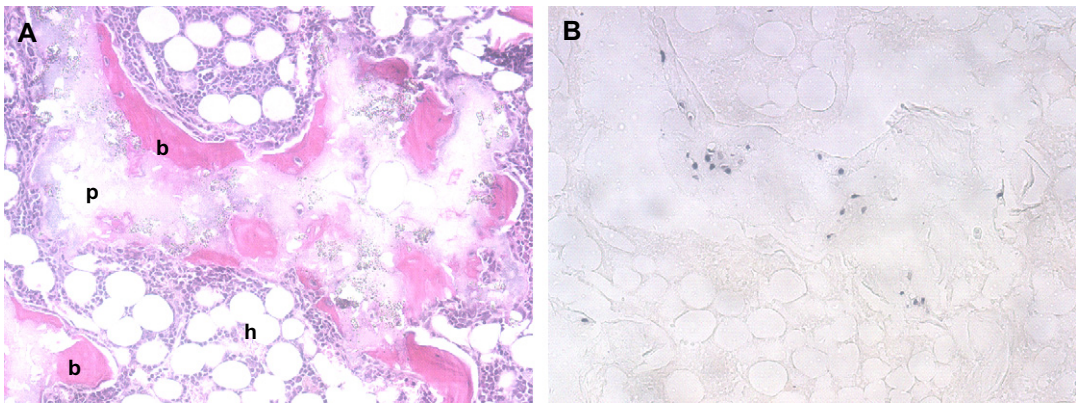

Figure 5. **(A)** 9-week-old, 3.0-million-BMSC transplant. **(B)** Adjacent section from transplant in (A). *alu*$^+$ cells, identified by in situ hybridization, are localized to the peri-osseous surfaces, consistent with osteoblasts, and to embedded osteocytes. No *alu*$^+$ cells among the hematopoietic elements. b, bone; f, fibrous connective tissue; p, particle; h, hematopoietic tissue. Magnification: 10×. Stain: Hematoxylin and eosin; paraffin embedding following demineralization.

Discussion

Hematopoiesis, the formation of blood cells, exclusively occurs in the bone marrow compartment in adult humans. Successful hematopoiesis depends upon an active marrow stroma, which provides a supportive microenvironment and from which growth factors are expressed [21,22]. Marrow stroma is also integral to the maintenance of bone, via differentiation of stromal osteoprogenitor cells into osteoblasts [4,21]. Because of stroma's dual role in supporting both hematopoiesis and bone formation, stromal activity must be examined in the context of both marrow and osseous function. Likewise, a study of bone formation by stromal osteoprogenitor cells without a concomitant study of hematopoiesis provides only half the story. For this reason, we became interested in the mechanisms by which BMSCs develop a hematopoietic compartment in conjunction with newly formed bone.

The purpose of this study was to test whether the formation of hematopoiesis-supporting stroma along with new bone in BMSC transplants is the product of a sufficiently mature and large bone environment. BMSCs of increasing dosage were transplanted with a standard aliquot of HA/TCP particles, and the amount of bone in each transplant was scored. The entire cross-section of each transplant was visualized, rather than just isolated high-power fields, because the irregular shapes of the particles and transplants introduces a high degree of variability from one high-power field to the next. A semi-quantitative scale of bone formation rather than histomorphometry was utilized in this study for ease of analysis. This scale has been validated by a direct comparison to histomorphometry [14]. When the bone scores reported on this scale have been compared to histomorphometric measurements of tissue sections, a correlation has been observed between the bone score and the

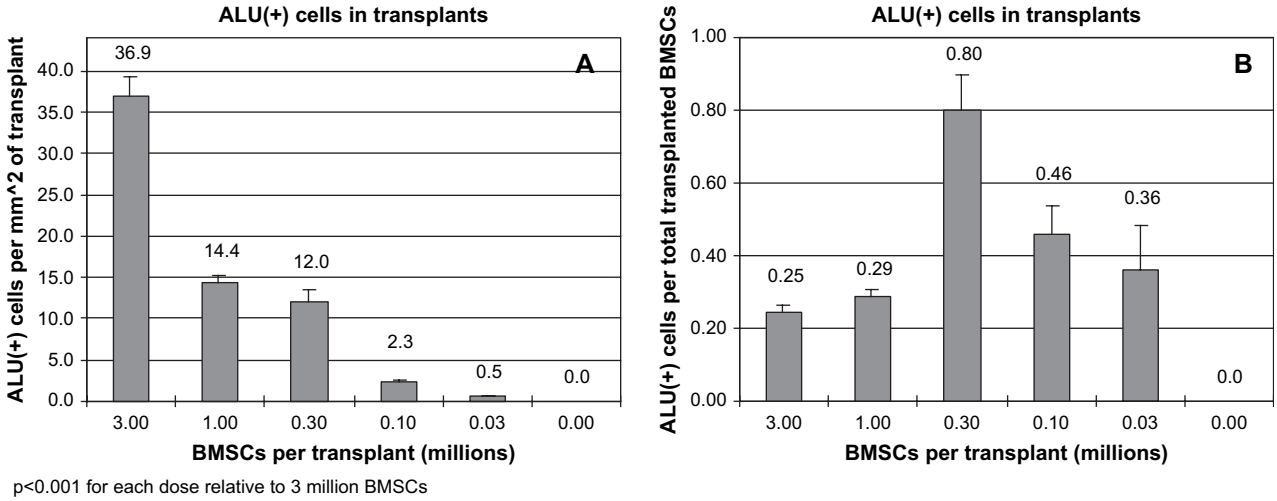

Figure 6. **(A)** *alu*$^+$ cells increase with increasing BMSC dosage, among 9-week-old transplants. **(B)** Comparison of the total *alu*$^+$ cells for each dosage with the original numbers of BMSCs in the transplant. Transplants with 0.3 million cells retained the highest proportion of *alu*$^+$ cells at 9 weeks (80%).

square root of the fraction of bone area to total transplant area (B/T) (r = 0.973) [14]. Additionally, nude mice served as recipients of the human BMSC transplants, because in our extensive experience of human BMSC xenogeneic transplants into nude mice, we have not seen any evidence of an immunologic response by the host against the transplanted cells.

Our results demonstrate that bone score increased with increasing BMSC dosage, and increased abruptly from 0.1 to 0.3 million BMSCs per transplant. Hematopoiesis was present only in transplants with significant bone formation, and all hematopoietic areas were surrounded by BMSC-derived trabeculae in occasional conjunction with HA/TCP particles. Additionally, the number of clusters of BMSCs recovered per HA/TCP particle increased linearly with increasing BMSC dosage. In 9-week-old transplants, the number of *alu*-positive cells in the transplants increased in a nonlinear fashion with increasing BMSC dosage, such that transplants with 0.3 million BMSCs retained the highest proportion of *alu*-positive cells relative to the original dose.

While we originally had expected that higher numbers of transplanted BMSCs would lead to more bone formation, the dependence of bone score on transplanted cell number turned out to be far from linear. Rather, it is in agreement with a sigmoid curve that consists of three distinct parts. First, in transplants with between 0 and 0.1 million BMSCs, bone formation is minimal without any hematopoiesis, and an increase in BMSC number is not accompanied by a corresponding rise in bone formation (lower lag phase). Within the 0.1 million to 1.0 million BMSC interval, bone formation increases rapidly with larger cell numbers, consistent with a steeply linear portion of the curve. Once a moderate amount of bone has formed, it is accompanied by hematopoiesis. A further increase in BMSC number beyond 1.0 million is followed by a minimal to no increase in bone or hematopoiesis formation (upper lag phase, or plateau).

Taken together, until a density threshold of about 0.3 million BMSCs per 40 mg aliquot of HA/TCP particles is reached, bone and hematopoiesis formation is negligible. A threshold value for bone formation is consistent with a report by Tavasolli, in which only those pieces of rat marrow that weighed greater than 15 mg formed bone upon subcutaneous transplantation; as transplant size increased beyond 15 mg, the weight of 5-week-old transplants increased in a linear fashion [23]. Likewise, the upper lag phase of the curve observed here agrees with results by Friedenstein's group [24]. When pieces of mouse femoral marrow had been transplanted under the renal capsule, increases in the size of transplanted tissue failed to yield proportional increases in the size of the mature transplants after 10 weeks. The authors suggested that when the numbers of transplanted osteogenic cells exceed the capacity of the transplantation site, a fraction of the cells forms a surplus, or reserve, of bone-forming cells.

Our interpretations of these observations are the following:

1. At transplantation, BMSC numbers on HA/TCP particles are proportional to their loading dose.
2. A reduction in BMSC number occurs in all transplants during bone formation, but this reduction is greatest at low and high doses. In low-dose transplants, low numbers of BMSCs result in negligible bone formation and heightened cell loss. In high-dose transplants, far more BMSCs are present than are needed for full bone formation, and excess cells are lost or preserved. In contrast, at medium dosages (0.3 million BMSCs per transplant) sufficient BMSCs are present for robust bone formation, and the majority of transplanted cells are recruited for this purpose.
3. Hematopoiesis occurs in conjunction with mature bone formation.
4. A minimal dose for optimum bone formation by human BMSCs is between 0.3 and 1.0 million cells per 40-mg transplant.

To date, no study has been undertaken to establish the minimum numbers of BMSCs necessary for adequate bone and hematopoiesis formation. However, Lennon and Caplan demonstrated that when a mixture of dermal fibroblasts and BMSCs was transplanted, at least 75% osteoprogenitor cells was necessary for bone formation within HA/TCP cubes 6 weeks following transplantation [25]. Because the authors described cell concentrations in suspension rather than cell numbers in the transplants, because they did not discuss hematopoiesis, and because it is not clear how many cells from the suspension were introduced to the cubes, it is difficult to compare their results to ours.

We were interested in determining whether the bone and hematopoiesis formation at 8 weeks was a good predictor of transplant organization at later time periods, or whether bone and hematopoiesis formation increased significantly after 8 weeks. Our comparison of bone formation during three time periods (8 weeks, 12 months, and 29 months) showed no significant difference in the amount of bone formed as a function of transplant period, suggesting that the amount of new bone increases only minimally after 8 weeks. Interestingly, however, the histologic appearance of the transplant continued to evolve beyond 8 weeks. While the overall shape and size of the transplant remained unchanged, the particles continued to undergo resorption and much of their volume was replaced by hematopoietic elements. The bone itself maintained its trabecular, laminar structure, and increasingly appeared to form a cortical shell at the periphery of the transplant. Because these transplants underwent minimal mechanical stress, the morphology of this thin bone likely reflects the low level of mechanical loading.

In summary, we have established that the formation of a mature bone organ, including the presence of

hematopoiesis, requires a sufficiently high local density of bone, which in turn requires a sufficient number of transplanted BMSCs. In addition to providing insight into the behavior of BMSCs, this study offers quantitative information on which cell dosage may be a suitable starting point for therapeutic human BMSC transplantation.

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